



Sex differences in scent-marking in captive red-ruffed lemurs.

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Title: Sex differences in scent-marking in captive red-ruffed lemurs.

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Abstract

Primate chemical communication remains underappreciated, as primates are considered to rely on other sensory modalities. However, various lines of evidence suggest that olfaction plays an important role in primate societies, including the conspicuous scent-marking behavior of many strepsirrhines and callitrichines. Although lemurs typically show scent-marking, little is known about this behavior in red-ruffed lemurs (*Varecia variegata rubra*). We combined behavioral observations and semiochemistry analyses to improve our understanding of scent-marking in two captive troops housed at Dudley and Twycross zoos (UK). We collected olfactory behavioral observations by focusing on two family troops ($N=7$) for 132 h. We investigated the volatile compounds of ano-genital scent-marks using solid-phase microextraction and gas chromatography-mass spectrometry and compared volatile chemical profiles with features of the signaller. Males scent-marked most frequently and predominantly in specific meaningful areas of the enclosure, while within females the occurrence of scent-marking was related to their age. We found behavioral sexual dimorphism, with male predominantly depositing secretions via neck and mandible glands and females via ano-genital glands. We identified a total of 32 volatile components of ano-genital gland secretion, including compounds that have already been found in other mammals as sex pheromones and cues to fitness, in ano-genital scent-marks spontaneously left on filter paper by adult females. Our findings suggest that red-ruffed lemurs might use scent-marking to convey information about sex and female age, with male neck-marking behavior playing defensive territorial functions and ano-genital marking related to socio-sexual communication.

Key-words: Communication, Signalling, Olfaction, Gas Chromatography–Mass Spectrometry, *Varecia variegata rubra*

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Introduction

Communication plays a fundamental role within animal societies, especially for species displaying complex social systems. The ultimate goal of communication is to spread information that influences the behaviors of receivers (Seyfarth and Cheney 2003). Animals can use various sensory modalities to transfer their messages to other individuals. In particular, olfactory communication is exhibited by several mammal species (reviewed in Scordato and Drea 2007), such as rodents (*e.g.*, Hurst *et al* 1998; Roberts 2007), but also by reptiles (*e.g.*, Muller-Schwarze 2006) and birds (*e.g.*, Leclaire *et al* 2017). Odor secretions are directly linked to the physiological conditions of senders (Harris *et al* 2018) and can be costly to produce (Scordato and Drea 2007), thus they are expected to deliver a more honest signal compared to other forms of communication (Hasson 1997).

Primates have traditionally been considered to be “microsmatic”, relying more upon other sensory modalities than olfaction (Dulac and Torello 2003). Since vision and acoustics are considered to be the main sensory modalities in most primate species little is known about the chemical signals used by non-human primates (Walker 1998). However, several studies support the hypothesis that chemical communication is crucial also for primates (*e.g.*, Porter and Moore 1971; Geissman and Hultegger 1994; Wedekind *et al* 1995; Wedekind and Furi 1997; Smith *et al* 2001; Jacob *et al* 2002; Hayes *et al* 2004, 2006; Heymann 2006; Knapp *et al* 2006; Laidre 2009; Smith 2006; Scordato *et al* 2007; Setchell *et al* 2010, 2011; Vaglio *et al* 2016). Particularly, it is established that some primates also rely heavily on olfaction in addition to vision and auditory senses; for instance, this is the case of squirrel monkeys (Laska *et al* 2000) and several lemurs (Gould and Overdorff, 2002; Scordato and Drea 2007). Strepsirrhines have retained their olfactory complexity (reviewed in Hayes *et al* 2006) due to morphological constraints that limit the visual signals produced by senders (Scordato and Drea 2007).

Lemur behavioral repertoire comprises both olfactory investigative and scent-marking behaviors. Lemurs show both direct and indirect olfactory investigative behaviors (Drea 2015); direct investigations may include behaviors such as sniffing and/or licking a conspecific's skin (palms, soles, eyelids, or nostrils) or genitals, and self-sniffing genitals, while indirect behaviors consist of sniffing and/or licking scent-marks deposited by the signaller. Scent-marking behavior is shown by several terrestrial vertebrates, including mammal and reptile species (Müller-Schwarze 2006; Roberts 2007). Scent-marks may include species-wide pheromones (*i.e.*, chemical substances released by an animal or insect which can affect a conspecific individual; for further details see Vaglio *et al* 2018) as well as highly individual odors. Scent-marking is a very effective form of communication within habitats that make difficult the detection of visual and auditory signals (Gould and Overdorff 2002), which is the case with forests inhabited by lemurs (Sussman *et al* 2003). In particular, this behavior is reasonably common in lemurs and New World monkeys – among which may play several functions, including the reproductive suppression of subordinate females, advertisement of individual “quality”, preparing males to assist in the delivery and care of newborn infants, and territorial defence (*e.g.*, Gould and Overdorff 2002; Pochron *et al* 2005; Heyman 2006) – while is less commonly reported in Old World monkeys and apes (*e.g.*, Freeman *et al* 2012). Especially, among strepsirrhines, social complexity may have selected for olfactory complexity in lemurs (del-Barco-Trillo *et al* 2012).

Mammals have a common pattern of scent-marking: glandular secretions, if not feces or urine, are placed at meaningful places such as along paths and territorial boundaries (Gosling and Roberts 2001). Scent-glands have been observed in various lemur species, including all *Eulemur* species (del-Barco-Trillo *et al* 2012), ring-tailed lemurs (Scordato and Drea 2007), red-bellied lemurs (Gould and Overdorff 2002), red-fronted lemurs (Hayes *et al* 2006), Milne-Edward's sifakas (Hayes *et al* 2004), black-and-white and red-ruffed lemurs (Gould

and Overdorff 2002). In particular, red-ruffed lemurs have multiple scent-glands (Gould and Overdorff 2002), composed of neck and mandible glands (male), and anogenital glands (male and female) (Pereira *et al* 1988); indicating that olfactory communication should be significant for this species (Elisa *et al* 2004).

The red-ruffed lemur is a large, frugivorous lemur species (Vasey 2006), which inhabit the residual primary forests of the Masoala Peninsula (Andriaholinirina *et al* 2014).

Red-ruffed lemurs have a variable social system; in smaller home ranges their group size is usually estimates between 2-5 individuals, whereas larger home ranges have been known to support between 18-32 individuals (Rigamonti 1993). Although red-ruffed lemur communities are not cohesive units, the home range is communally defended. In addition, only females participate in communal home range defense against females from other groups, which includes agonistic behaviors such as chasing, scent-marking, vocalizing, and even physical contact with members of neighboring communities (Vasey 2005; 2007). Females are dominant to males, winning almost all agonistic encounters with them and rarely showing submissive behavior towards them (Raps and White 1995; Meyer *et al* 1999). Communication is commonly observed as vocalisations, emitting species-specific calls which serve several functions and are transmittable between groups (Macedonia and Taylor 1985); however, also chemical communication is thought to play a crucial role in group dynamics (Elisa *et al* 2004).

The overarching aim of this study is to improve our understanding of the role played by chemical communication, particularly focusing on scent-marking behavior, in red-ruffed lemurs. We predict that red-ruffed lemurs advertise information about their sex, age and rank by using scent-marking. We also anticipate that this study may contribute to further exploring the connection between functional and mechanistic levels of lemur scent-marking (Charpenter *et al* 2010).

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119 **Materials and Methods**

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121 *Subjects and Housing*

122 We studied two captive troops of red-ruffed lemurs ($n=7$) housed at Dudley and
123 Twycross zoos (UK). The troop housed at Dudley Zoological Gardens consisted of two
124 related (brothers) adult males (13 years old) and one unrelated adult female (12 years old).
125 The troop housed at Twycross Zoo consisted of one adult male (11 years old), one adult
126 female (12 years old) and their offspring (two 1.5 years old females). Red-ruffed lemurs are
127 considered sexually mature at 2 years old, with first conception approximately one year later
128 (Vasey 2007). Adult females were contracepted, and all individuals in non-breeding season
129 (i.e. regarding red-ruffed lemurs in captivity in the Northern Hemisphere breeding usually
130 occurs in December-January with births in April-May; Brockman *et al* 1987).

131 We carried out behavioral observations and odor sampling from September to
132 November 2016 (Twycross Zoo) and from July to September 2018 (Dudley Zoological
133 Gardens). In both institutions, the troops lived in an indoor enclosure (heated to 28°C) with
134 access to an outdoor enclosure ('visitor walkthrough' enclosures).

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136 *Ethics Statement*

137 This study followed the guidelines for the care and use of captive animals in the
138 UK, involving non-invasive methods for obtaining both behavioral data and odor samples
139 from red-ruffed lemurs. Moreover, the study was conducted in compliance with the
140 Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES)

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141 and approved by the Life Sciences Ethics committee at the University of Wolverhampton
142 (UK) and the Ethics committees at Dudley Zoological Gardens and Twycross Zoo (UK).

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144 *Behavioral Data Collection and Analysis*

145 We collected behavioral data by instantaneous scan sampling (Altmann 1974), with
146 behaviors recorded at 30-sec intervals over the duration of an hour in three time periods (two
147 during the morning, and one during the afternoon), two days per week, over three months.
148 Alongside the scan sampling we also used *ad libitum* sampling for recording olfactory
149 behaviors (**Table I**), including both scent-marking (ano-genital marking, neck-marking – that
150 is scent-marking via neck and mandible glands) behavior and locations (comprising of
151 “hatches”, “tree branch”, “indoor enclosure”, “wooden hut”, and “climbing frame”). We
152 recorded a total of 132 hours of observations throughout the study period, including 360 scan
153 samples each sampling day on the entire group.

154 We investigated the relationships between individuals and scent-marking behavior in
155 relation to sex and age of senders. We also investigated the role played by different types of
156 scent-marking behavior, and locations of scent-marks within the enclosure. We tested all
157 variables through ~~both~~ Shapiro-Wilk ~~and Kolmogorov-Smirnov~~ tests for normality. As data
158 were not normally distributed, we performed non-parametric Kruskal-Walis tests followed
159 by pairwise Mann-Whitney U *post-hoc* tests. All tests were carried out using SPSS v.23, and
160 a significance level of $P<0.05$ was applied.

161
162 [insert table I here]

163
164 *Odor Sampling and Analysis*

We collected odor secretions spontaneously released via scent-marking by red-ruffed lemurs on brand-new filter paper fixed on hatches, climbing equipment, and tree trunks and branches (**Figure I**). Unfortunately, we were not able to collect any odor sample from males, while we collected scent-marks deriving from ano-genital marking by all the females (14 samples, 3-4 replicates per individual). In addition, we placed control filter paper in the environment to control for the contact with wood (where there may be chemical compounds deriving from the wood, but also algae, microorganisms, etc.) and we exposed control filter paper also to the air during sampling in order to detect any chemical compounds which did not derive from the red-ruffed lemurs. We collected odor samples immediately after scent-mark deposition by red-ruffed lemurs in the outdoor enclosure. We placed all samples and controls into brand-new sterile vials (Supelco) and immediately stored them at -20°C. We used 10-ml screw-capped clear glass vials (thread: 18O.D. 22.5-mm x H 46-mm) closed by teflon-faced rubber septa and seals (1.3-mm thick).

We conducted laboratory analyses at the Rosalind Franklin Science Centre, University of Wolverhampton (UK). We investigated the volatile components of odor secretions using established solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) and applying the same methods used in our previous work on mandrill odor signals (Setchell *et al* 2010; Vaglio *et al* 2016).

We introduced a 65- μ m polydimethylsiloxane/divinylbenzene SPME syringe needle through the vial septum and then we exposed the fibre to the headspace above the sample in the vial for 15 minutes at 40°C. We analysed the adsorbed volatile analytes of all samples by using a 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) EI, 70 eV, coupled directly to a 7890B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a fused silica HP5-MS UI capillary column (Agilent Technologies,

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3 189 Santa Clara, CA, USA) 30 m x 0.25 mm crossbonded 5%-phenyl-95%-dimethylpolysiloxane,
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5 190 film thickness 0.25 μm . We maintained the injector and transfer line temperatures at 270°C
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8 191 and 280°C, respectively. We made injections in splitless mode (purge valve opened after 1
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10 192 min) with a constant flow of helium carrier gas of 1 mL min⁻¹. We started the oven
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12 193 temperature program at 45°C for 2 min, then raised it by 4°C min⁻¹ to 170°C, and finally by
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14 194 20°C min⁻¹ to 300°C.

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18 195 We assessed potential contamination due to the lab environment through blank
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20 196 analyses of an empty 10-mL vial (Supelco) following the same procedure as for the samples.
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23 197 In addition, we conditioned the fibre at 260 °C pre- and post- injection, for 5 and 20 mins
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25 198 respectively in order to avoid any possible carry-over effects.

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28 199 We tentatively identified eluted compounds by comparing the experimental spectra
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30 200 with the spectra provided by the mass-spectral library in ChemStation (Agilent Technologies,
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33 201 Santa Clara, CA, USA) and NIST (National Institute of Standards and Technology) Database,
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35 202 version MSD F.01.01.2317 (Agilent Technologies, Santa Clara, CA, USA). We accepted a
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37 203 putative identification when the minimum matching factor was higher than 80%. If more than
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39 204 one compound was a good match for the same GC peak then we considered the
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42 205 chromatographic retention time and compared it with the retention time reported in the
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44 206 literature for the same chromatographic column type (El-Sayed 2016) in order to minimize
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46 207 the chance of misidentification. We created a data matrix using the peak area relative to each
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48 208 identified compound by using the integrated signal of the deconvoluted total ion current
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50 209 (TIC). We analysed all samples in a short period of time (approximately 24 hours) to
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53 210 minimize interassay variability. We removed all the contaminants (*i.e.*, any compounds that
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56 211 appeared in the ‘environmental controls’ and ‘lab blanks’) from the scent-mark results.

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[Insert Figure I here: Filter paper attachments]

Results

Behavioral Observations

During the study period olfactory behaviors were exhibited predominantly by males (40.00%), followed by adult females (34.48%) and subadult females (25.52%). These behaviors included both scent-marking (ano-genital marking and neck-marking) and investigative behaviors (sniffing and/or licking an area within the enclosure, sniffing and/or licking a conspecific, self-licking of the ano-genital glands). Scent-marks were most commonly deposited by males (51.55%), followed by adult females (26.80%) and subadult females (21.65%) (**Figure II**), although differences were not significant between the sexes ($U=137.5$; $p=0.688$).

[Insert Figure II here: Frequency of marks in relation to sex and age]

We observed males ($N=3$) scent-marking significantly more via neck glands rather than via ano-genital glands ($U=12.5$; $p<0.001$). Females ($N=4$) displayed the opposite behavioral pattern; showing a significantly higher frequency of ano-genital marking rather than neck marking ($U=41.5$; $p=0.022$). We found significant differences in neck-marking behavior between individuals, and particularly between males and females ($N=7$; $U=12.5$; $p=0.003$).

We found a significant difference ($N=7$; $Z=-5.675$, $p<0.001$) in scent-marking behaviors between inside and outside locations, with 81.73% of scent-marks occurring in the

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237 outdoor enclosure. Moreover, scent-marks were most commonly deposited near, or upon, the
238 hatches leading to the indoor and off-show enclosure (18.27%). However, scent-marks were
239 also deposited on tree branches next to the path of the walk-through, all climbing frames in
240 the walk-through, a hut providing shelter within the walk-through, and upon furniture in the
241 indoor enclosure.

242 We also found a preference of location when considering the type of scent-marking
243 performed; “hatches” were used most commonly overall for neck-marking (81.82%)
244 compared to ano-genital marking (18.18%), whereas “climbing frame” was used more for
245 ano-genital marking (71.43%) than neck-marking (28.57%).

246 We found significant differences in areas used for neck-marking, most commonly
247 upon “hatches” ($X^2=23.152$; $p<0.001$), and upon “tree branches” ($X^2=9.456$; $p=0.009$).
248 Deposition of neck scent-marks upon “hatches” was significantly different between males
249 and females ($U=04.5$; $p<0.001$). Neck-marking on “tree branches” showed a difference
250 between males and females ($U=30.0$; $p=0.029$), and between adult and subadult females
251 ($U=30.0$; $p=0.029$).

252 We found significant differences in areas used for ano-genital marking, with most
253 frequent occurrences upon “hatches” ($X^2=11.748$; $p=0.003$) and “climbing frame”
254 ($X^2=13.119$; $p<0.001$). Deposition of ano-genital marks upon “hatches” was significantly
255 different between adult and subadult females ($U=84.0$; $p=0.037$). Ano-genital marks upon
256 “climbing frames” were also significantly different between adult and subadult females
257 ($U=35.0$; $p<0.001$).

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259 *Odor secretions*

We identified a total of 32 individual compounds from the analysis of 14 filter paper samples of female ano-genital secretions. These compounds included a range of naturally occurring odorous volatile compounds such as hydrocarbons, terpenes, terpene alcohols and ketones. Tentative identifications are listed in **table II**, while typical chromatograms (1 from the blank control and 12 from a female lemur ano-genital scent-marks) are shown in **figure III**. There was variation in the number and abundance of the compounds observed from sample to sample across different individuals. However, six compounds (benzaldehyde, 2-ethyl-1-hexanol, p-cresol, cis-p-mentha-2,8-dien-1-ol, 2-pinen-4-one, pentadecane) were present in all samples. We were not able to analyse the ratios of individual components in order to compare the volatile profiles with features of the signaller (for instance, adult vs subadult females) due to the small amount of filter paper samples.

[Insert Table II here. Secretion compounds]

[Insert Fig. III here. Chromatographs]

Discussion

Primates rely on olfactory communication in several contexts, including foraging, territorial defense, individual and family recognition, mate choice and mother-offspring bonding (Zeller 1987). Although very little is known about Old World primates, research has been accumulating on chemical communication in strepsirrhines and New World monkeys; particularly, semiochemical data are accessible for few non-human primate species, including various strepsirrhines [galago (Crewe et al 1979); lemurs (delBarco-Trillo *et al* 2011, 2012; Hayes *et al* 2004, 2006; Palagi and Dapporto 2006; Scordato *et al* 2007), owl monkeys (Macdonald *et al* 2008), marmosets and tamarins (Epple *et al* 1993; Smith *et al* 2001),

284 macaques (Curtis *et al* 1971) and mandrills (Setchell *et al* 2010, 2011; Vaglio *et al* 2016)]. In
285 this study we focused on scent-marking behavior, via both behavioral and chemical
286 approaches, in two troops of zoo-managed red-ruffed lemurs.

287 Red-ruffed lemurs, as the well-studied ring-tailed lemurs, are characterised by a
288 female-dominated society. In ring-tailed lemurs males scent-mark more than females
289 (Pochron *et al* 2005) and female age is positively correlated to scent-marking frequency
290 (Kappeler 1990; Gould and Overdorff 2002; Pochron *et al* 2005). Similarly, in our study,
291 males scent-marked significantly more than any female and adult females showed the highest
292 frequency of scent-marking within females. Therefore, our findings support the prediction
293 that scent-marking would differ between individuals based on their sex, as found in other
294 lemur species such as sifakas (Pochron *et al*. 2005), and age, as found in ring-tailed lemurs
295 (Kappeler 1990; Gould and Overdorff 2002; Pochron *et al*. 2005).

296 Neck-marking was exhibited by all study subjects, but significantly more by males. In
297 contrast, females exhibited ano-genital scent-marking significantly more than males. This
298 supports the hypothesis of sexual dimorphism in red-ruffed lemur scent-marking, as already
299 observed by Vasey (2003). The preferences shown by males for neck-marking “hatches” and
300 ano-genital marking “tree branches”, and by adult females for ano-genital marking “climbing
301 frames”, confirm behavioral sexual dimorphism. These observable preferences also suggest
302 that scent-marking behaviors might play different roles in males and females, as observed in
303 other primates, including ring-tailed lemurs (Scordato and Drea 2007), black-and-white
304 ruffed lemurs (Pereira *et al* 1988) and mandrills (Vaglio *et al* 2016).

305 Our results also support the hypothesis that scent-marking might have a territorial
306 function in this species (Pereira *et al* 1988). In particular, males scent-marked mostly specific
307 meaningful places, by using neck-marking for hatches (small openings allowing access from

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3 308 outdoor to indoor enclosure; *i.e.*, potential role of territorial defense) and ano-genital marking
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5 309 for tree branches and climbing equipment (areas of frequent transit by individuals; *i.e.*,
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8 310 potential role of social communication). This also implies that scent-marks released via neck-
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10 311 marking and ano-genital marking might communicate different messages to the receivers by
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12 312 conveying information about distinct features of the senders. Previous studies have indicated
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14 313 information regarding sex to be conveyed in scent-marks from ring-tailed lemurs (Hayes *et al*
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16 314 2004, Scordato *et al* 2007), but absent in odorants from sifakas (Hayes *et al* 2004, 2006).

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20 315 Although scent-marking behavior is observable, it is challenging to decipher the
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22 316 message which is chemically communicated. Therefore, the chemical investigation of odor
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24 317 secretions released by scent-marking is critical to understand the message transferred by this
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26 318 behavior. Since we used spontaneously released scent-marks, we were able to investigate
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28 319 odor secretions released by scent-marking and thus corresponding to the exact message sent
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30 320 by red-ruffed lemurs.

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34 321 A total of 32 compounds were tentatively identified within the ano-genital secretions
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36 322 released by female study subjects (excluding ~~environmental and control compounds~~, lab
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38 323 contaminants ~~as well as~~ co-eluted compounds). This low amount of volatile compounds in
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40 324 comparison to other female lemur ano-genital marks (for example, ring-tailed lemurs and
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42 325 sifakas; Hayes *et al* 2004, Scordato *et al* 2007) might be explained by the fact that breeding
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44 326 *versus* non-breeding season (Scordato & Drea 2007) and chemical contraception (Crawford
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46 327 *et al* 2011) can have significant impacts on semiochemical signals in lemurs. For instance, in
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48 328 ring-tailed lemurs (Crawford *et al* 2011) genital odorants of adult contracepted females were
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50 329 proved altered, including decreased richness, modified relative abundances, and minimized
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52 330 individual chemical distinctiveness of their volatile chemical profiles.
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3 331 Volatile hydrocarbons have previously been identified in odorants deriving from ring-
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7 333 monkeys such as mandrills (Setchell *et al.* 2010; Vaglio *et al.* 2016) and olive baboons (Vaglio
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9 334 *et al.* in preparation). In particular, high-molecular weight volatile hydrocarbons might act as
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11 335 a fixative which slows the release of more volatile compounds, as suggested for major
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13 336 urinary proteins in mice (Green 2016; Hurst *et al.* 1998). The compounds benzaldehyde, p-
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15 337 cresol (also known as p-methylphenol), hexanal and geranylacetone are commonly
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17 338 encountered in mammal scent markings (e.g., lions, wild dogs, wolves, mice, red foxes)
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19 339 (Osada *et al.* 2015; Roberts *et al.* 2010; Soso & Koziel 2016). The compound benzaldehyde
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21 340 has already been found in gland secretions released by marmosets (Smith *et al.* 2001), and
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23 341 functions as sex pheromone in other mammals (reviewed in El-Sayed 2016) and also as cue
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25 342 to genetic quality (reviewed in Wyatt 2014) in other vertebrates (e.g., in the crested auklet, a
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27 343 seabird with citrus scent based on decanal and octanal produced by both sexes during the
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29 344 breeding season, concentration correlates with rank in males). Thus, benzaldehyde might
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31 345 serve as pheromone and play a role in signalling individual quality also in red-ruffed lemurs.
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33 346 In addition, ethyl-phenol occurs in rat urine as mate attraction signal and also in beaver urine
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35 347 as part of a multicomponent signal of range occupation (reviewed in Apps *et al.* 2015). The
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37 348 compound 2-pine-4-one (also known as verbenone) is a bark beetle antiaggregation
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39 349 pheromone (Lindgren & Miller 2002), which has similarly been found in other insects (i.e.,
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41 350 bees and butterflies) and is also naturally occurring in plants (reviewed in Bakthavatsalam
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43 351 2016). 2-ethyl-1-hexanol and cis-p-metha-2,8-dien-1-ol both appear to be associated with
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45 352 fragrances. Finally, other compounds, such as α -pinene, are known to derive from plants;
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47 353 therefore, they might be a by-product and potentially vary with the environmental context but
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49 354 could also contribute to the message communicated by red-ruffed lemurs through scent-
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51 355 marking (for instance, convey information about group identity).
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357 Conclusions

358 In conclusion, the present study supports the hypotheses (Smith *et al* 2015) of sexual
359 dimorphism and of more than one function served by scent-marking in red-ruffed lemurs. We
360 suggest that scent marking could serve a function in intergroup spacing and intrasexual
361 competition for both sexes, as might be expected in a female-dominant species.

362 In particular, male neck-marking might have a defensive territorial function while
363 ano-genital marking might play a role in socio-sexual communication in this lemur species.
364 Furthermore, our findings suggest that odor secretions released via ano-genital-marking
365 might convey information about the age of female signallers. Additionally, the similarity of
366 red-ruffed lemur's volatile chemical profiles to those found in other vertebrates would
367 support our previous suggestion (Setchell *et al* 2010) that non-human primates are not as
368 microsmatic as traditionally considered.

369 Since this study is based on seven animals living in two captive family troops it can
370 only be considered a preliminary work for the red-ruffed lemur species. Future research work
371 should focus on a larger sample size, record behaviors consistently throughout the day, and
372 investigate the odor secretions released by adult non-contracepted females and also by male
373 scent-marks. In addition, it would be crucial to study the perception by the recipient, for
374 instance looking for evidence of behavioral or physiological responses facilitated by scent-
375 marks via bioassay tests (Wyatt 2014). Also, more detailed analysis of the ratios of individual
376 components could form the basis of further studies. Finally, although we focused on the
377 volatile profile of red-ruffed lemur odor, we also recognize the significance of non-volatile
378 components of odor secretions, as high-molecular weight compounds may extend the
379 persistence of volatile signals in scent-marks (Alborne 1984; Belcher *et al* 1990; Hurst and

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Beynon 2004).

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Table I. Ethogram (based on Scordato and Drea, 2007, and Vaglio *et al* 2016, modified).

Behavior	Description
Scent-Marking; Neck / Mandible	Individual rubs neck region against substrate or upon an item within the enclosure
Scent-Marking; Ano-genital	Individual rubs genital region against substrate or upon an item within the enclosure
Sniffing / Licking; Environment	Individual deliberately places nostrils or tongue within 3cm from substrate or an item within the enclosure and sniffs/licks
Sniffing / Licking; Conspecific	Individual deliberately places nostrils or tongue within 3cm from a conspecific and sniffs/licks
Self-Licking	Individual uses tongue to lick an area near a scent gland on their own body

Table 2 – Volatile compounds present in filter paper samples from female lemur anogenital secretions identified tentatively using ChemStation and NIST mass spectral databases (v. MSD F.0101.2317). Compounds in bold font were found in all samples.

Retention Time (mins)	Tentative Compound ID	Molecular Weight
3.906	Hexanal	100
6.057	5-methyl-3-hexanone	114
7.413	Alpha-pinene	136
8.077	1-isopropyl-4-methylenebicyclo[3.1.0]hex-2-one	134
8.268	Benzaldehyde	106
8.623	3,7,7-trimethyl-1,3,5-cycloheptatriene	134
9.096	Phenol	94
9.269	6-methoxy-5-hepten-2-one	126
10.720	2-ethyl-1-hexanol	130
12.362	p-Cresol	108
12.553	cis-Verbenol	152
13.385	cis-p-Mentha-2,8-dien-1-ol	152
14.104	1,7,7-Trimethylbicyclo[2.2.1]hepta-2-one	152
14.536	L-Pinocarveol	152
14.791	trans-Verbenol	152
15.605	p-Ethyl-phenol	122
15.928	Terpinen-4-ol	154
16.415	Alpha-Terpineol	154
16.615	Myrtenol	152
17.047	2-Pinen-4-one	150
18.252	Carvone	150
19.217	p-Mentha-1,8-dien-3-one	150
23.283	4,7,7-Trimethylbicyclo[4.1.0]hept-3-ene-2-one	150
23.443	Tetradecane	198
25.094	Geranylacetone	194
25.899	Isomethylionone	206
26.513	Pentadecane	212
30.871	2,6,10-Trimethylpentadecane	254
32.208	Heptadecane	240

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32.372	2,6,10-Trimethylhexadecane	268
34.446	n-Tetracosane	338
34.591	2,6,10,14-Tetramethylhexadecane	282

For Peer Review



Fig. 1. Sterile filter paper attached to popular scent-marking locations using plastic cable ties.
(a) hatches, (b) climbing frame equipment, (c) tree branches.

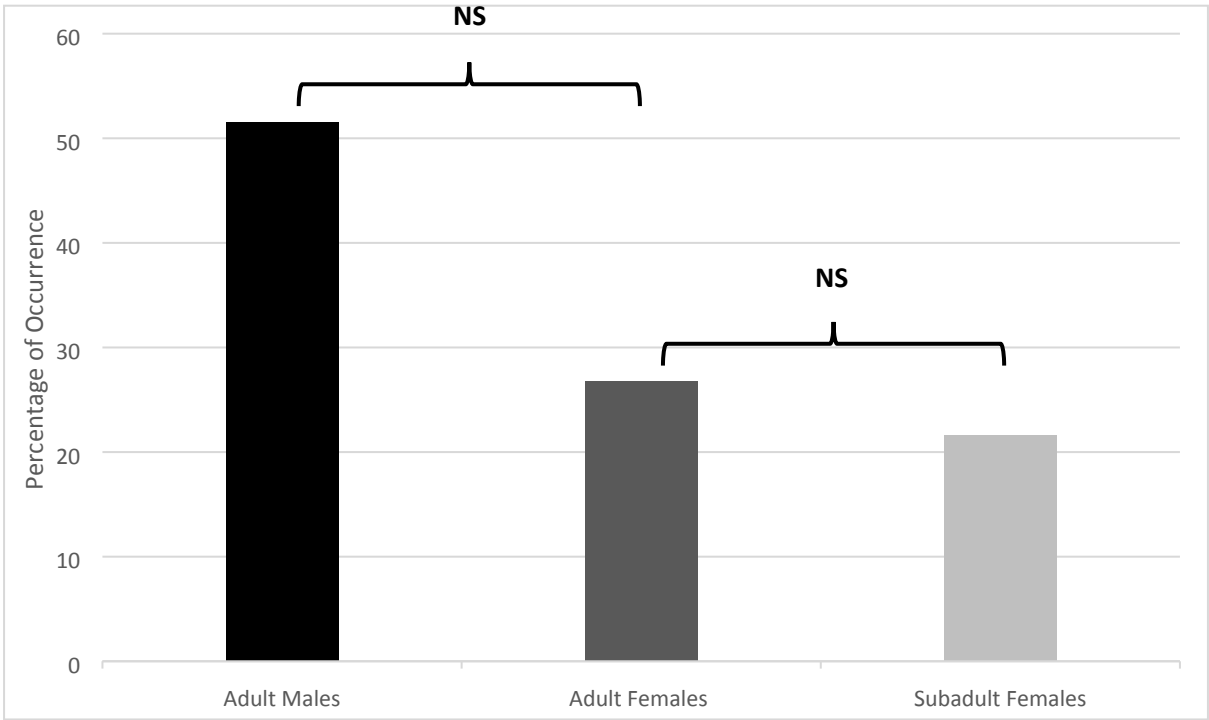


Fig. II. Percentage of occurrence for total scent-marks associated to classes of authors. Males scent-marked the most (51.55%), followed by adult females (26.80%) and subadult females (21.65%), however differences were not statistically significant.

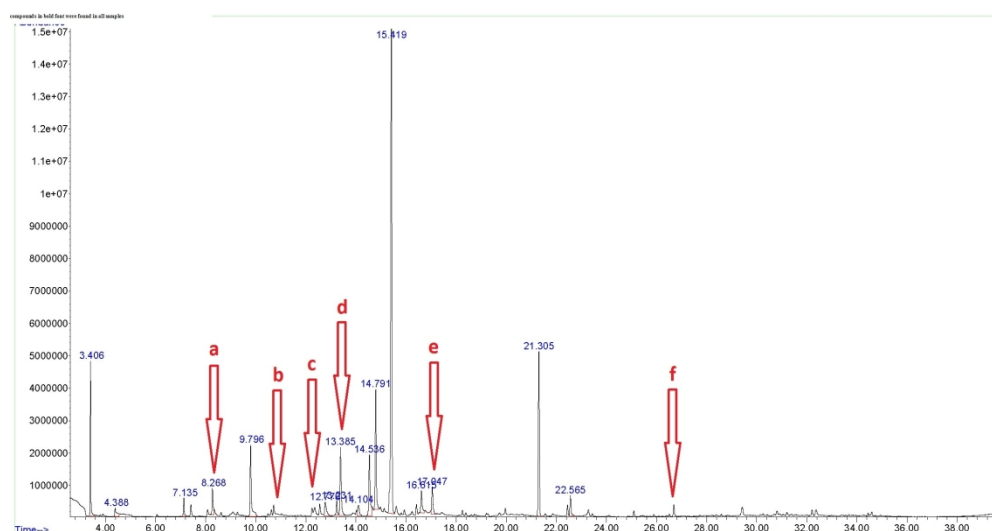


Fig III. Example chromatograms from (a) one adult female ano-genital odour secretions, showing contaminants and meaningful biological compounds. Red arrows indicate the six meaningful biological compounds which were found in all samples: (a) benzaldehyde; (b) 2-ethyl-1- hexanol; (c) p-cresol; (d) cis-p-mentha-2,8-dien-1-ol; (e) 2-pinen-4-one; (f) pentadecane.

283x149mm (200 x 200 DPI)

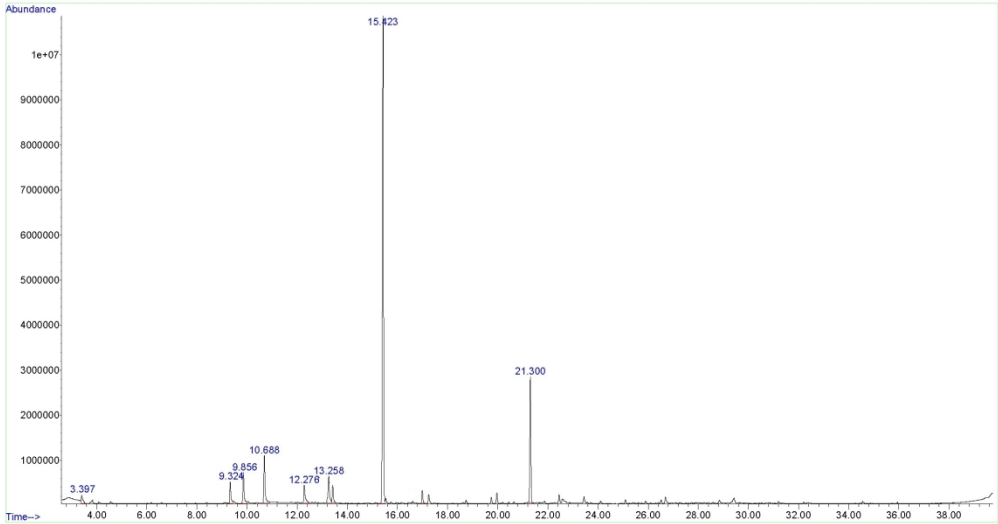


Fig III. Example chromatograms from (b) the control sample, showing contaminants.

283x149mm (200 x 200 DPI)

Research Highlights

- Red-ruffed lemurs use scent-marking behaviors to convey information about sex, female age and rank.
- They show sexual dimorphism in scent-marking behavior.
- Neck-marking plays defensive territorial functions and ano-genital marking is related to socio-sexual communication.

For Peer Review



A red-ruffed lemur at Twycross Zoo

863x1151mm (72 x 72 DPI)